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Molecular Genetic Resources for Development of 1% Linolenic Acid Soybeans

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ABSTRACT

Advanced plant breeding will incorporate the most efficient methods available to introgress new traits and develop improved crops. Molecular markers that are specifically targeted to desirable alleles are important molecular genetic resources for selection of traits. Reducing the amount of linolenic acid in soybeans [*Glycine max* (L.) Merr.] is a desired breeding objective so that oxidatively stable soybean oil can be produced without the production of *trans* fatty acids. The objective of this work was to determine the molecular genetic basis for soybeans containing 1% (10 g kg^{-1}) linolenic acid in the seed oil fraction and to develop molecular markers specific for identified alleles. Utilizing the soybean homologs of *Arabidopsis FAD3* as candidate genes, mutations were discovered in all three *GmFAD3* genes in the soybean line A29. The mutations were associated with the linolenic acid phenotype in segregating populations. Molecular markers specific for the mutant alleles enabled capture of the phenotype. Novel combinations of mutant alleles at the three *GmFAD3* loci allowed the development of new germplasm containing 1% linolenic acid in the seed oil along with SNP-based molecular markers that can be used in a backcross breeding strategy.

The impetus for developing soybean cultivars with lower linolenic acid concentration in the oil fraction of the seed is the need to rapidly respond to emerging needs related to production of healthful foods. For human nutrition, the fatty acid profile of foods has received considerable attention in recent years with generalized warnings concerning the amount of saturated fats and *trans* fatty acids in the diet. As part of an effort to reduce consumption of ingredients that increase the risk of coronary heart disease, the addition of *trans* fatty acid content to food labels begins in 2006. The need to improve oxidative and flavor stability of typical soybean oil results in the production of *trans* fatty acids as a consequence of chemical hydrogenation of polyunsaturated fatty acids. Linolenic acid content of the oil is responsible for the oxidative instability and flavor problems in soybean oil (Dutton et al., 1951; Lui and White, 1992), and development of soybean containing reduced linolenic acid concentrations is a current breeding goal.

Breeding based on chemical phenotype for single seed composition traits has been successful in lowering linolenic acid from wild-type levels of 60 to 100 g kg^{-1} oil to as low as 10 g kg^{-1} oil for lines containing three independent mutations (Fehr et al., 1992; Rahman et al., 1998; Ross et al., 2000). Because linolenic acid content in soybean is controlled by multiple genes, a more efficient breeding strategy would be to use molecular markers for rapid introgression of the trait in a backcross breeding strategy to develop elite lines (Bilyeu et al., 2005). Molecular markers for the lowered linolenic acid trait would also allow the combination of multiple traits in one line where a backcrossing strategy could be utilized to capture mutant alleles of a low linolenic acid donor parent with a recurrent parent that has other desirable seed composition traits with or without associated molecular markers.

Genetics of the lowered linolenic acid trait have received considerable attention. After the original *fan* mutation corresponding to lower linolenic acid levels was detected in line C1640 (Wilcox and Cavins, 1985; Wilcox and Cavins, 1987), other independent loci (*fan2*, *fan3*, and *fanx*) were also identified in lines with further reductions in linolenic acid content (Fehr et al., 1992; Rahman et al., 1998; Ross et al., 2000). In an effort to provide a molecular understanding of the low linolenic acid trait, we identified and characterized the soybean homologs of the *Arabidopsis* omega-three fatty acid desaturase gene, *FAD3* (Bilyeu et al., 2003; Yadav et al., 1993). At least three versions of this gene exist in the soybean genome, *GmFAD3A*, *GmFAD3B*, and *GmFAD3C*, and *GmFAD3A* was shown to be *Fan* (Bilyeu et al., 2003; Anai et al., 2005). Recently, we determined the molecular genetic basis for the low linolenic acid soybean line CX1512–44, which had mutant alleles of both *GmFAD3A* and *GmFAD3C* (Bilyeu et al., 2005). The *GmFAD3A* gene was shown to have a greater impact on seed linolenic acid levels than *GmFAD3C*, consistent with higher expression of *GmFAD3A* in developing seeds (Bilyeu et al., 2005; Bilyeu et al., 2003).

The lowest level of linolenic acid reported for a soybean line is 1% of the oil fraction (10 g kg^{-1} oil) described for line A29 (Ross et al., 2000). A29, which is not related to other low linolenic acid lines with reported molecular information (CX1512–44, J18, M5, and M24 [Anai et al., 2005; Bilyeu et al., 2005]), was developed by combining three independent mutations: *fan* from line A5 (Hammond and Fehr, 1983), *fan2* from A23, and *fan3* from a mutagenized derivative of line A89–144003 (Ross et al., 2000). The *fan* mutation in line A5 is caused by a *GmFAD3A* deletion (Bilyeu et al., 2003; Byrum et al., 1997). The very low linolenic acid lines A29, IA3017, and IA3018 are related by pedigree (W. Fehr, personal communication, 2005). The objective of this research was to investigate the *FAD3* genes in soybean line A29 to

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Abbreviations: PCR, polymerase chain reaction; SNP, single nucleotide polymorphism.

identify possible mutations and determine if the mutant alleles were associated with the 1% linolenic acid trait.

MATERIALS AND METHODS

Cloning and Sequence Analysis

Primers were designed based on the sequences deposited in GenBank to amplify soybean *GmFAD3A* (AY204710), *GmFAD3B* (AY204711), and *GmFAD3C* (AY204712) cDNAs or genomic DNA from line A29. Reverse transcriptase reactions, PCR (polymerase chain reaction) amplification, isolation of products from agarose gels, and cloning were as previously described (Bilyeu et al., 2003).

Plant Growth

The low linolenic acid soybean lines A29 (10 g kg^{-1} oil), IA3017 (10 g kg^{-1} oil), and IA3018 (26 g kg^{-1} oil) were chosen as candidates for potential *FAD3* gene mutations. Low linolenic soybean lines A29, IA3017, and IA3018 were provided by Dr. Walter Fehr, Iowa State University (Ross et al., 2000). The low linolenic acid line 2721 (30 g kg^{-1} oil) was an F_3:F_6 line produced from a cross between 'Pana' and CX1512–44. Line 2721 was homozygous for the CX1512–44 mutant alleles of *GmFAD3A* and *GmFAD3C* (Bilyeu et al., 2005).

SS97–6946 is an experimental breeding line with normal fatty acid profiles (77 g kg^{-1} oil), resistance to multiple soybean cyst nematode HG types, and appropriate maturity (D. Sleper, unpublished data, 2003). A cross of SS97–6946 × IA3017 produced F_1 seeds in the summer of 2003 at the Bradford Research and Extension Center (BREC), located near Columbia, MO. F_1 plants were grown in Guacima, Costa Rica, and F_2 seed was chipped for fatty acid analysis with the remainder of the seed planted and grown in Nazareth, Costa Rica, during the fall of 2003 and spring of 2004. Chips were shipped to BREC and analyzed for linolenic acid concentration. Single-plant threshed F_2:F_3 seed of lines containing the lowest linolenic acid content (18 lines containing up to linolenic acid concentrations of 15 g kg^{-1} oil) was returned to BREC for planting progeny rows in summer 2004. Because no lines were recovered which were homozygous for all three mutations, five individual aabbcc F_2:F_3 plants derived from an aabbCc F_2 parent were used as pollen donors for a SS97–6946 × F_2:F_3 backcross (SS97–6946 × [SS97–6946 × IA3017]). The resulting three confirmed BC_1F_1 seeds were germinated in moist germination packets (Mega International, St. Paul, MN) and transferred to soil for growth in controlled chambers set at 27.5/23°C day/night with 14.5 h daylength at a light intensity of 750 μmol m^{-2} s^{-1}. Eighty seeds were produced, and fatty acid and genotype data were collected for 61 individuals.

Seeds of a A29 × 2721 cross were produced in Costa Rica in 2003. F_1 (1 seed), F_2 (62 seeds), and F_2:F_3 seeds were germinated in moist germination packets and transferred to soil for growth in controlled chambers set at 27.5/23°C day/night with 14.5 h daylength at a light intensity of 750 μmol m^{-2} s^{-1}. Twelve lines were identified in the F_2 generation to have homozygous mutant alleles for *GmFAD3B* and *GmFAD3C*, and a subset (8) of those lines were followed to the F_3 generation to test for segregation of *GmFAD3A* alleles.

Genotype Analysis

Detection of mutant alleles followed the procedure as described by Bilyeu et al. (2005) with PCR amplification of the genomic region encompassing the single nucleotide polymorphism (SNP) followed by restriction enzyme digestion of products. When primer sequences are indicated, the forward primer is listed first, and all primers are listed in the 5′ to 3′ orientation.

For the A29 *GmFAD3B* allele assay, amplification primers were B932: AGCCACAGAACTCACCATCAA and IABrev: TGGCACAGTGAATCTAATG. Following amplification conditions as described for the CX1512–44 alleles (Bilyeu et al., 2005), products were digested with 2.5 U *HpaI* (New England Biolabs, Beverly, MA) per reaction and resolved on agarose gels. Wild-type alleles produced fragments of 196 and 162 bp while A29-derived mutant alleles produced a single 358-bp fragment.

For the A29 *GmFAD3C* allele assay, amplification primers were IACleft: TTGGATCAACAACATTCACCA and IACright: CATCACATGTTTGTGGTCTTGA. Following amplification, products were digested with 5 U *BccI* (New England Biolabs) per reaction and resolved by McSNP analysis (Ye et al., 2002) or on agarose gels. Wild-type alleles produced fragments of 199 and 56 bp while A29-derived mutant alleles produced a single 250-bp fragment.

For the *GmFAD3A* deletion assay, a Taqman assay was used in a reaction with primers that detected both the *GmFAD3A* gene (3AD1/3AIX amplification primers [Bilyeu et al., 2005]; dual-labeled probe [FAM/BHQ1] WTprobe: TCGCGGCAAGGTAACAAAAA) and the *PEPC16* gene (PEPC16f: TTCCTTTATCAGAAATAACGAGTTTAGCT, PEPC16r: TGTCTCATTTTGCGGCAGC; dual-labeled probe [VIC/BHQ1] PEC16probe: CCCTCCCCTGTACCCATGTTTCCATTATAA [Tuteja et al., 2004]). Reactions for PCR included 0.33 μ*M* of each amplification primer, 0.21 μ*M* of each dual labeled probe, and 1× QuantiTect Probe PCR mix (Qiagen Inc., Valencia, CA). PCR conditions were 95°C for 12 min, 44 cycles of 95°C for 20 s, 59°C for 20 s, and fluorescence read. In samples for which there was amplification of the *PEPC16* target, fluorescence was scored for the amplification of the *GmFAD3A* target.

Templates for all genotype PCRs consisted of 2-mm washed FTA (Whatman, Clifton, NJ) card punches prepared from leaves according to the manufacturer's instructions. Genomic DNA was isolated for control and parental lines either using the DNeasy Plant Mini Kit (Qiagen, Inc.) or as described previously (Bilyeu et al., 2003) and used at 5 to 50 ng per PCR reaction.

Phenotype Analysis

Fatty acid analysis was conducted as described for seed chips or whole crushed seed (Beuselinck et al., 2006; Bilyeu et al., 2005). For the F_2:F_3 seed fatty acid determination, three or five F_2:F_3 seeds were crushed and analyzed individually. The concentration of linolenic acid in the seed sample was determined as a percentage of the total fatty acids of extracted oil by lipid gas chromatography of fatty acid methyl esters.

RESULTS AND DISCUSSION

Identification of Mutant FAD3 Alleles and Molecular Marker Assays

We characterized the soybean *FAD3* genes as candidates for potential mutations from the low linolenic acid soybean lines A29 (10 g kg^{-1} oil), IA3017 (10 g kg^{-1} oil), and IA3018 (26 g kg^{-1} oil). Our previous work identified the *fan* mutation in line A5 as a deletion of *GmFAD3A* gene sequences, which presumably is a null allele (Bilyeu et al., 2005; Bilyeu et al., 2003). We determined that the 1 and 2% linolenic acid lines A29, IA3017, and IA3018 also have deletions of the *GmFAD3A* gene (data not shown). For genotyping assays, either simulta-

neous amplification of both the *GmFAD3A* and *GmFAD3B* genes or an allele-specific Taqman assay can be used to detect the deletion of *GmFAD3A*, as previously described (Bilyeu et al., 2005; Bilyeu et al., 2003; see materials and methods). Although unambiguous identification of homozygous mutant individuals is robust, neither detection method distinguishes heterozygous individuals from homozygous wild-type lines.

When cDNA from line A29 was initially used as template for RT-PCR, the *GmFAD3B* gene reactions failed to produce a product. Therefore, genomic DNA from line A29 was used to amplify the *GmFAD3B* gene region, and the product was cloned, sequenced, and compared to the partial *GmFAD3B* genomic sequence present in GenBank (accession AX088031). Among several minor differences in intron sequences, a SNP was identified in the 3′ splice site consensus (Brown et al., 1996) sequence preceding exon 4 (Fig. 1). Improper splicing was subsequently detected in mRNA from this region that would lead to a frameshift and premature termination of the protein product (data not shown). An assay was designed to distinguish wild-type and mutant alleles of *GmFAD3B* based on the presence or absence of a *HpaI* restriction enzyme site for wild-type and A29 derived mutant alleles, respectively (Fig. 1C).

The *GmFAD3C* gene was amplified by RT-PCR from A29 cDNA. After cloning and sequencing of *GmFAD3C*, a SNP was discovered (C910T) that resulted in a H304Y mutation (Fig. 2) in the histidine-rich region II of the protein sequence (Shanklin et al., 1994). The altered histidine residue corresponds to one shown to be essential for desaturase activity (Shanklin et al., 1994). An assay was designed to distinguish between wild-type and A29 derived mutant alleles at *GmFADC* based on the presence or absence of a *BccI* restriction enzyme site for wild-type and A29-derived mutant alleles, respectively (Fig. 2B).

Both 1% linolenic acid lines A29 and IA3017 were found to have the same set of *GmFAD3A*, *GmFAD3B*, and *GmFAD3C* mutant alleles. The related line IA3018, which contains 2.6% linolenic acid grown under field conditions, was genotyped and found to contain the *GmFAD3A* and *GmFAD3C* mutations, but not the A29 allele of *GmFAD3B*. Thus, soybean lines selected for the lowest known linolenic acid concentration contain mutant alleles of three *GmFAD3* genes.

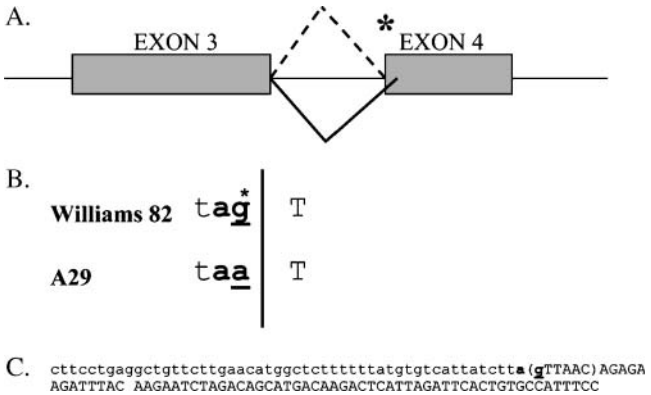

Fig. 1. Identification of a splice-site mutation in the A29 *GmFAD3B* allele. (A) Schematic diagram of *GmFAD3B* exons 3–4 (gray rectangles) connected by an intron (horizontal lines). Normal splicing and intron removal is represented by dashed lines above the introns. Abnormal splicing (solid lines below the introns) characterized A29 mRNA with activation of a cryptic splice site within exon 4 leading to exclusion of the first seven bases of exon 4. A mutation in the splice site was identified at the first base before exon 4 (position denoted by an asterisk). (B) Sequence comparison of Williams 82 and A29 *GmFAD3B* splice site junction at the intron 3–exon 4 boundary. PCR products generated from amplification of genomic DNA were cloned and sequenced. Sense sequence is listed 5′ to 3′, with the vertical line indicating the separation of intron 3 and exon 4. The final base of the intron in the A29 allele is changed from G to A (underlined), mutating a consensus splice site (AG-splice, bold [Brown et al., 1996]). (C) Sequence of genomic DNA from Williams 82 *GmFAD3B* beginning within the 3′ part of intron 3 and ending at base 533 of the cDNA sequence (exon 4 begins at base 466 of the cDNA sequence). Exon sequences are listed in capitals while intron sequences are lowercase. The base mutated in A29 is underlined, the *Hpa*I recognition sequence (GTTAAC) is in parentheses, and the consensus splice sequence is in bold.

Using Molecular Markers in a Backcross Breeding Strategy

Part of our interest in developing molecular markers for the low linolenic acid trait is to provide the tools to accelerate introgression of traits into elite soybean cultivars. The use of molecular markers supercedes the need for extensive chemical analysis and facilitates the capture of the trait in a backcrossing program that can rely on selection of unrelated traits. Toward this end, we initiated a cross between SS97–6946 (D. Sleper, unpublished data, 2003) and IA3017. Following the production of F_1 seed, a typical breeding strategy was used in which F_1 plants were grown, allowed to self-fertilize, and the F_2 seed was chipped for fatty acid analysis. Agronomic selections and selections based on fatty acid analysis were made on F_2 plants. As an initial genotyping screen, two individuals from each F_2:F_3 family were genotyped. No F_2:F_3 families were identified that contained all three homozygous mutant alleles of the *FAD3* genes. However, individual plants were identified by genotype to be homozygous mutants, and these individual homozygous mutant F_2:F_3 plants were used as pollen donors in backcrosses to SS97–6946.

Fig. 2. Identification of a mutation in the A29 allele of *GmFAD3C*. (A) Amino acid alignment of a portion of the soybean and *Arabidopsis* FAD3 protein sequences (At2 g29980, GenBank accessions AY204710, AY204711, and AY204712). Identical amino acids are highlighted in black while similar amino acids are highlighted in gray. The wild-type and A29 GmFAD3C alleles are shown from amino acid 280 to 329. A SNP in the coding sequence results in a H304Y mutation (indicated above the alignment with an asterisk) for A29 GmFAD3C. Histidine-rich region II (Shanklin et al., 1994) is underlined. (B) Sequence of genomic DNA from Williams 82 *GmFAD3C* beginning at base 864 and ending at base 951 of the cDNA sequence. Only exon sequences are in the listed region. The base mutated in A29 is underlined (base 910 of the coding sequence) and the *Bcc*I recognition sequence is in parentheses.

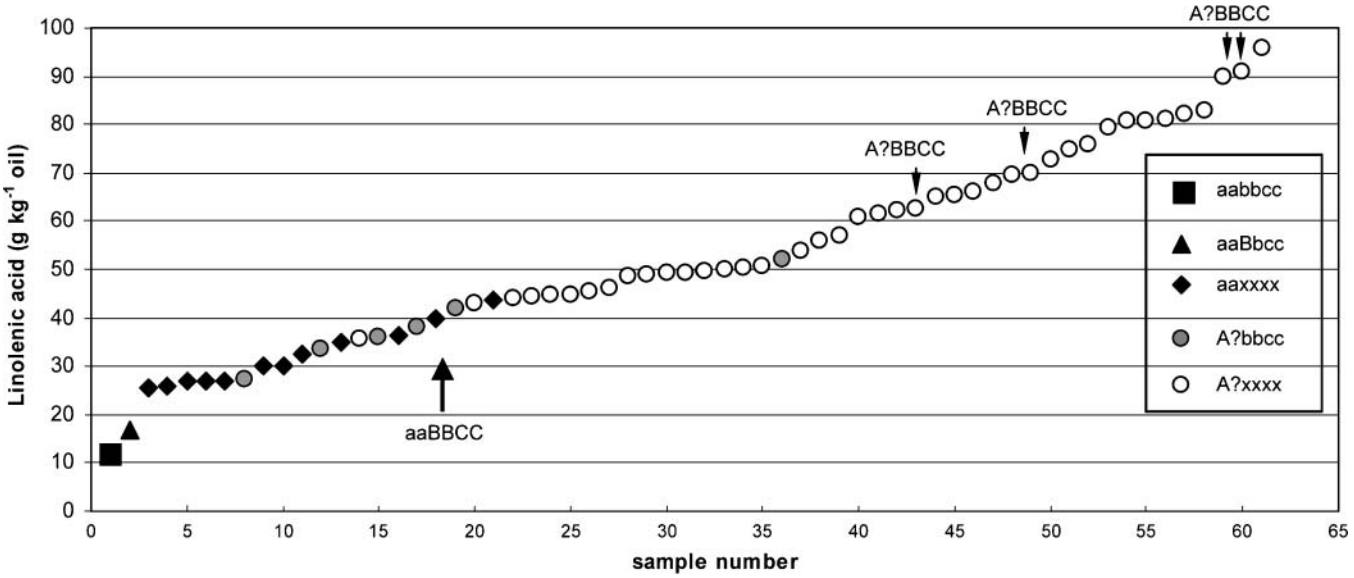

Fig. 3. Association of linolenic acid phenotype with *FAD3* genotype in segregating BC_1F_2 seeds derived from a backcross (SS97–6946 × [SS97–6946 × IA3017]). Sixty-one BC_1F_2 seeds were chipped for fatty acid analysis with the remainder of the seed germinated and then subjected to genotyping for A29 or wild-type alleles of *GmFAD3A*, *GmFAD3B*, and *GmFAD3C*. It was not possible to distinguish Aa from AA genotypes. Samples were organized by increasing linolenic acid concentration. Black diamonds (♦) represent soybean lines containing homozygous *GmFAD3A* mutant alleles and any combination of wild-type or mutant *GmFAD3B* and *GmFAD3A* alleles (aaxxxx). Circle symbols (○) represent soybean lines either heterozygous or homozygous wild-type for the *GmFAD3A* allele (Aa or AA, designated A?). The black square (■) represents the only line with the complete homozygous mutant genotype (aabbcc). The black triangle (▲) represents the only line with a genotype of aaBbcc. No lines were identified which had the alternate genotype, aabbCc. Circles with gray shading indicate those lines which had either Aabbcc or AAbbcc genotypes (A?bbcc). Other selected genotypes are identified with arrows, and the genotype is listed.

The association of genotype and phenotype was assessed in field grown plants derived from a single cross (SS97–6946 × IA3017). F_2:F_4 seeds produced on field grown selfed F_2:F_3 plants of selected plants with known genotypes were tested for fatty acid composition. While the recurrent parent germplasm contained an average of 7.7% linolenic acid (77.0 g kg^{-1} oil), the five F_2:F_3 lines identified with the homozygous mutant genotype for all three genes produced F_2:F_4 seed with an average of 1.3% linolenic acid (13.0 g kg^{-1} oil). Grown in the same field environment, IA3017 produced seeds with an average of 1.2% linolenic acid (12.0 g kg^{-1} oil).

Over the subsequent cycle of backcross breeding (SS97–6946 × [SS97–6946 × IA3017]), plants were grown in controlled environment chambers. BC_1F_1 plants (derived from SS97–6946 × F_2:F_3) were confirmed using molecular markers. The BC_1F_2 seeds were chipped for fatty acid analysis and germinated for further growth and genotyping (Fig. 3). One BC_1F_2 line was recovered with the homozygous mutant genotype for all three *GmFAD3* genes, and it contained the lowest concentration of linolenic acid (12 g kg^{-1} oil). Because 27 genotypes are possible from the segregation of the three *GmFAD3* loci, and the assay for the *GmFAD3A* deletion does not distinguish wild-type from heterozygous individuals, the association of genotype and phenotype is not entirely clear. In general, samples homozygous for the *GmFAD3A* deletion produce the lowest linolenic acid phenotype, regardless of the genotype at *GmFAD3B* and *GmFAD3C*. In addition, mutations in *GmFAD3B* and *GmFAD3C* also appear to additively contribute to lowered linolenic acid levels as demonstrated by the relatively low linolenic acid phenotype for samples with at least one copy of a wild-type *GmFAD3A* allele in combination with homozygous mutations in *GmFAD3B* and *GmFAD3C* (Fig. 3, samples 8, 12, 15, 17, 19, and 36, gray circles). Conversely, samples with at least one copy of a wild-type *GmFAD3A* allele in combination with homozygous wild-type *GmFAD3B* and *GmFAD3C* contained some of the highest linolenic acid levels.

When BC_1F_2:F_3 seeds from a BC_1F_2 plant with the *GmFAD3A* deletion but still segregating for both the *GmFAD3B* and *GmFAD3C* mutant alleles were ana-

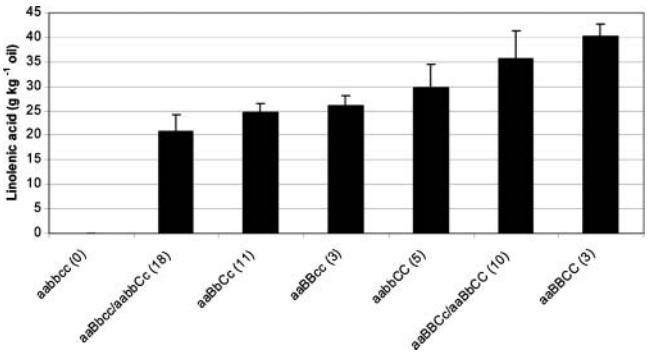

Fig. 4. Association of linolenic acid phenotype with genotype in plants segregating for mutant alleles of *GmFAD3B* and *GmFAD3C* derived from a backcross (SS97–6946 × (SS97–6946 × IA3017)). Fifty $BC_1F_{2:3}$ seeds produced on a aaBbCc plant (26 g kg^{-1} of oil) were chipped for fatty acid analysis with the remainder of the seed germinated and then subjected to genotyping for A29 or wild-type alleles of *GmFAD3B* and *GmFAD3C*. The samples were pooled by genotype, as listed. The number of lines represented in each pool follows the genotype in parentheses. No lines were recovered with the complete mutant genotype. Histograms represent mean linolenic acid (g kg^{-1} of oil) plus one standard deviation from the mean.

Table 1. Listing of available mutant alleles at the three soybean *GmFAD3* loci with perfect molecular markers developed from four low linolenic acid soybean lines.

Mutant line	Linolenic acid g kg^{-1} oil	Desaturase genes and alleles			Reference
		GmFAD3A	*GmFAD3B*	*GmFAD3C*	
A5	40	aa^{29} (deletion)†	BB	CC	Bilyeu et al., 2003
CX1512–44	28	aa^{cx} (SNP)	BB	cc^{cx} (SNP)	Bilyeu et al., 2005
IA3017	10	aa^{29} (deletion)	bb^{29} (SNP)	cc^{29} (SNP)	this work
C1640	40	aa^{C1640}(SNP)	BB	CC	Chappell and Bilyeu, 2006

† Mutant genotype for three *GmFAD3* genes. Superscript designates source of mutant allele derived from a mutant soybean line; 29 = A29 and cx = CX1512–44. Uppercase designates wild-type alleles.

lyzed for fatty acid content, additional evidence for an association between mutant alleles and low linolenic acid phenotype was observed (Fig. 4). The individual contribution of wild-type alleles of *GmFAD3B* and *GmFAD3C* to linolenic acid phenotype cannot be determined with confidence from this limited dataset, but additional experiments support nearly equivalent contributions for *GmFAD3B* and *GmFAD3C* that are smaller than the *GmFAD3A* contribution to linolenic acid levels when substituting wild-type and mutant alleles (data not shown).

Five BC_1F_2:F_3 seeds derived from the single BC_1F_2 plant containing all three mutations were analyzed for fatty acid profile. The phenotype of the BC_1F_2:F_3 seeds, which theoretically contain 75% of the recurrent parent genome, was a linolenic acid average of 1.2% (12.0 g kg^{-1} oil). A discrepancy was noted in the linolenic acid phenotype of seed chips when compared to the phenotype of the remainder of the same crushed seed; the linolenic acid level of the seed chips averaged 12 g kg^{-1} higher than the crushed seed. Whole crushed seeds provide a complete sample to more precisely determine the true phenotype, but eliminate the possibility to regenerate the individual genotype (i.e., the seedling).

The association data presented here supports a model in which all three *GmFAD3* genes contribute to the total enzymatic capacity to produce linolenic acid in the seed oil. Consistent with our earlier work, the mutation in the *GmFAD3A* gene described here caused a greater reduction in linolenic acid concentration than mutations in *GmFAD3B* or *GmFAD3C* (Bilyeu et al., 2005; Bilyeu et al., 2003).

Recombination of Mutant Alleles

Together with the previously identified mutations in *GmFAD3A* and *GmFAD3C* present in the low linolenic acid lines C1640 and CX1512–44 (Bilyeu et al., 2005; Chappell and Bilyeu, 2006), there are now a total of six available mutant alleles with perfect (i.e., specific to the causative mutation) molecular markers at three soybean *GmFAD3* loci (Table 1). The mutant *GmFAD3* genotype in A29 can be designated aa^{A29}bb^{A29}cc^{A29} and distinguished from the CX1512–44 derived mutant genotype, designated aa^{cx}BBcc^{cx}. No mutations were identified in the CX1512–44 *GmFAD3B* gene (Bilyeu et al., 2005).

For crosses with A29 or IA3017 as a parent, the inability to distinguish lines heterozygous for the *GmFAD3A* mutant allele from lines with homozygous wild-type *GmFAD3A* alleles prevented the use of molecular markers for the most efficient backcrossing strategy. Because the CX1512–44 line contains a mutant allele of *GmFAD3A* that is the result of a SNP that can be assayed to easily distinguish the two homozygotes and the heterozygotes, it could be used as an alternative source of the *GmFAD3A* mutant allele (Bilyeu et al., 2005).

We initiated a cross between A29 (aa^{A29}bb^{A29}cc^{A29}) and 2721 (aa^{cx}BBcc^{cx}, Bilyeu et al., 2005), and analyzed progeny for genotype and phenotype in the F_2 and F_2:F_3 generations to select lines homozygous for different combinations of the three SNP-containing mutant alleles (aa^{cx}bb^{A29}cc^{cx} or aa^{cx} bb^{A29}cc^{A29}). As expected for segregation of only mutant alleles at *GmFAD3A* and *GmFAD3C* and mutant and wild-type alleles of *GmFAD3B*, the range of linolenic acid concentrations in F_2 seeds was narrow (12 to 51 g kg^{-1} oil). Two F_2 individuals (lines designated B1–52 and B1–62) were confirmed in the F_3 generation to be homozygous for both CX1512–44-derived mutant alleles and homozygous for the A29-derived *GmFAD3B* allele, aa^{cx}bb^{A29}cc^{cx}. One F_2 individual (designated line B1–15) had the alternate genotype, aa^{cx} bb^{A29}cc^{A29}. The linolenic acid contents for these lines are listed in Table 2, along with a line with the aa^{A29}bb^{A29}cc^{cx} genotype. These results indicate that the available mutant alleles can be substituted for each other and in combinations that enable SNP detection for all three *FAD3* genes as well as stable inheritance of the 1% linolenic acid phenotype.

CONCLUSIONS

Introgression of soybean seed composition traits into elite cultivars can be accomplished by utilizing chemical

Table 2. Linolenic acid phenotype in F_n generations of lines derived from the cross A29 (aa^{A29}bb^{A29}cc^{A29}) × 2721 (aa^{cx}BBcc^{cx}) with different genotype combinations of mutant *FAD3* alleles.

Generation	B1–40 (aa^{A29}bb^{A29}cc^{cx})†	B1–15 (aa^{cx}bb^{A29}cc^{A29})	B1–52 (aa^{cx}bb^{A29}cc^{cx})	B1–62 (aa^{cx}bb^{A29}cc^{cx})
	Linolenic acid g kg^{-1} oil			
F_2‡	14	13	12	13
F_3	15	13	16	15
F_4	Not determined	13	14	12

† Homozygous mutant genotype for three *GmFAD3* genes. Superscript designates source of mutant allele derived from a mutant soybean line; cx = CX1512–44.
‡ Chipped seed was used for fatty acid analysis in the F_2 generation otherwise analysis was performed on whole seed.

phenotyping, using molecular markers, or combinations of the two screening strategies. The lowered linolenic acid trait is controlled by multiple genes, with three of those genes shown to be the three fatty acid desaturase candidate genes described in this work, *GmFAD3A*, *GmFAD3B*, and *GmFAD3C*. For the lines A29 and IA3017, three soybean FAD3 genes had deleterious mutations. Relatively straightforward molecular marker assays were designed to distinguish these mutant alleles from their wild-type counterparts. Selection for the three mutations in early generations can achieve reductions in linolenic acid concentration from approximately 8% (80.0 g kg^{-1} oil) to below 2% (20.0 g kg^{-1} oil) with stable inheritance of the trait. The most efficient use of time and resources would need to be determined for each breeding program, but a combination of chemical phenotype screening followed by genotype selection in the F_2 generation using molecular markers would keep costs low while allowing complete capture of the trait. Either a backcrossing strategy could be used or screening in advanced generations could emphasize other traits.

The mutant alleles identified as part of this project were readily substituted with other mutant alleles of *GmFAD3A* and *GmFAD3C* that had been previously identified (Bilyeu et al., 2005). Breeding programs that have already incorporated a *fan* mutation (an allele of *GmFAD3A*) to lower linolenic acid concentration now have the option of accomplishing further reductions in linolenic acid by incorporating mutant alleles of *GmFAD3B* and *GmFAD3C*. In addition, the novel combinations of mutant *GmFAD3* genes that impart a 1% (10.0 g kg^{-1} oil) linolenic acid phenotype developed as part of this project provide new resources to the soybean breeding community.

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